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Partial 18S rDNA sequences in Taxodiaceae and their potential in phylogenetics

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PARTIAL 18S rDNA SEQUENCES IN TAXODIACEAE AND THEIR POTENTIAL IN PHYLOGENETICS

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ABSTRACT

PARTIAL 18S rDNA SEQUENCES IN TAXODIACEAE
AND THEIR POTENTIAL IN PHYLOGENETICS

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The sequence of the 3' end of the 18S gene in the ribosomal DNA cistron was assessed for its utility in the phylogenetic analysis of genera in the redwood (Taxodiaceae) family. PCR using primers NS-1 and NS-2, cycle sequencing and silver staining were chosen to amplify, sequence, and detect a 557 base-pair region of this gene. This system was optimized to produce DNA sequence analysis up to 250 bases in length.

Silver sequencing was found to be an accurate and reproducible method for sequencing the 18S gene in all six genera studied (*Sequoia sempervirens*, *Sequoiadendron giganteum*, *Cryptomeria japonica*, *Metasequoia glyptostroboides*, *Cunninghamia lanceolata*, and *Taxodium distichum*). Their sequences were highly conserved, with only one transition detected in *Metasequoia*. Due to the conserved nature of the gene, any taxonomic inferences were precluded. Future work will focus on the ITS-1 region of the ribosomal cistron, a region that is less conserved.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Taxodiaceae is an ancient coniferous family that can be traced back 175 million years to the middle of the Jurassic period. The modern members of this family are found in the fossil records from the Cretaceous period and represent lineages that evolved 100 million years ago (Miller 1977). Despite the age of Taxodiaceae, the family is made up of only 13 to 15 species grouped into 10 genera and include: *Sequoia sempervirens*, *Sequoiadendron giganteum*, *Cryptomeria japonica*, *Glyptrostrobus lineatus*, *Metasequoia glyptostroboides*, *Sciadopitys verticillata*, *Taiwania cryptomerioides*, *Cunninghamia konishii*, *C. lanceolata*, *Taxodium distichum*, *T. ascendens*, *T. mucronarum*, *Athrotaxis cupressoides*, *A. selaginoides*, *and A. laxifolia*. Although fossil evidence suggests that the genera of Taxodiaceae each had multi-continental distributions, the family now clings to isolated endemic populations. In fact, no modern genus of Taxodiaceae is found on more than one continent (Miller 1977). *Sequoia* and *Sequoiadendron* are now restricted to western North America while *Taxodium* is found only in eastern North America and Mexico. Additionally, the eastern Asian genera *Glyptrostrobus*, *Metasequoia*, *Taiwania*, *Cunninghamia*, and Japanese genera *Cryptomeria*, and *Sciadopitys* have been reduced from worldwide distribution. Also, the wide geographic range of *Athrotaxis* has been reduced to only Tasmania and it is now the only representative of Taxodiaceae in the Southern Hemisphere (Miller 1977).

Indeed, Taxodiaceae occupies a unique position within the conifer families because of the ancient divergence of the modern taxa and their subsequent isolation. These factors have also made their phylogeny more obscure and subject to debate. Morphological studies of Taxodiaceae have produced few characters common to the entire family. Most shared states are among subsets of the genera and this has prompted some authors to suggest that Taxodiaceae is a paraphyletic or polyphyletic taxon that is "a heterogeneous assemblage of

genera, united only by the absence of specialized states that have been used to define the remaining families of conifers" (Gadek and Quinn 1989). In fact, the only apparent unifying morphological character among the Taxodiaceae genera is the presence of a fused bract-scale complex in their ovulate cones (Florin 1951). Ironically, even this character is shared with the closely related Cupressaceae; a family that some believe is a monophyletic group of Taxodiaceae because of other shared characters e.g., *Metasequoia's* opposite leaves and the scale-like leaves of *Athrotaxis* (Eckenwalder 1976). Furthermore, there is growing evidence that *Sciadopitys* should be raised to the family level to form Sciadopityaceae (Schlarbaum and Tsuchiya 1984, 1985; Price and Lowenstein 1989; Brunsfeld et al. 1994).

Despite extensive morphological studies of Taxodiaceae, the family's phylogeny still remains in question because of subjective taxonomic considerations such as character importance or its derived state. Character prejudice has certainly contributed to the variable number of species ascribed to *Taxodium* and *Athrotaxis*; some investigators contend that *T. distichum* and *T. ascendens* are one polymorphic species (*T. distichum*) (Gadek and Quinn1989) and that *A. laxifolia* is a hybrid of *A. cupressoides* and *A. selaginoides* (Cullen and Kirkpatrick 1988). Such disputes are often caused by insufficient defining characters that are used to draw meaningful relationships among the groups being studied. To achieve a clearer understanding of the relationships within Taxodiaceae and its place in Coniferales, more characters need to be utilized. Indeed, many researchers have increasingly turned to chemical, biochemical, immunological, and genetic analysis. Although not all of these taxonomic techniques have been applied to the study of Taxodiaceae, many hold promise because of their use in related taxa.

One character that has been added to the analysis of Taxodiaceae is its chemical characterization. Chemical analysis is a tool that has been successfully used since the 1970's to characterize the Angiosperms (Stafford and Lester 1986). Recently, though, a

larger body of information has been collected from Gymnosperms and in particular Taxodiaceae. Gymnosperms in general, however, are more restricted in their variety of flavonoids. Nevertheless, Taxodiaceae does possess flavonoids unique to the family and they include the proanthocyanidins, prodelphenidins, and biflavones (Stafford and Lester 1986; Gadek and Quinn 1989). Using either 1-dimensional thin layer chromatography or 2-dimensional paper chromatography, relationships may be formed from flavonoid data (Stafford and Lester 1986). This technique does have its limitations in studying Taxodiaceae though; it requires that controversial primitive and derived chemical states be determined and is subject to seasonal and pathogenic variation (Stafford and Lester 1986). Because of such variation, no taxonomic conclusions were rendered concerning Taxodiaceae.

Unlike chemical studies, isoenzyme analysis has been used in the phylogenetic study of a wide variety of organisms from protozoans (Truc 1993) to mammals (Hartl 1990). It has, apparently, also been used to study Taxodiaceae at the generic level in a Chinese study for which only an English summary was available (Hu 1987). That study grouped *Sequoia* and *Sequoiadendron* with *Taxodium*, a genus whose placement has remained equivocal (Brunsfeld 1994). Isoenzymes are a taxonomic character whose heterogeneity within related groups of proteins can be electrophoretically or chromatographically resolved. Because of slight changes in proteins from different alleles, the related polypeptides migrate through various media at different rates. Identification of similar or novel alleles and measurement of electrophoretic mobility provide taxonomic characters. Isoenzyme analysis does have its limits and cannot address inconsistent evolutionary rates, convergent evolution, or problems in comparability of data sets (Hartl 1990). Because of such shortcomings, taxonomic research has increasingly turned to more quantifiable methods.

Immunological studies or radioimmunoassays (RIA) have been used to study the phylogeny of Taxodiaceae as well as other plants, algae, and mammals (Price and

Lowenstein 1989). RIA analysis of Taxodiaceae produced phylogenies that are consistent with morphological studies for most of the family. The assay uses antibodies to assess evolutionary differences in protein structure. The strength of the assay is that it is able to detect small differences in protein morphologies. The greater the difference in binding affinity or specificity between an antibody and two proteins, the wider the inferred evolutionary distance. In fact, RIA analysis of the Pinaceae family, a taxonomically less disputed relative of Taxodiaceae, has shown strong agreement with traditional morphological treatments of the family (Price and Lowenstein 1989). Also, unlike chemical analysis, the taxonomic characterization of proteins by RIA is concentration independent and provides data that are not environmentally influenced. Nevertheless, the assay is limited in its ability to analyze proteins because of variable antibody production in the sera of the injected animal. This limits the control of the proteins being studied, and the inability to characterize specific proteins could certainly raise doubts about the data compatibility between different taxonomic groups. Certainly, some sets would select antibodies to proteins that would be more amenable to ordinal relationships than specific ones. A concern more relevant to the data quality is the fact that RIA only detects changes in the 3-dimensional structure of the protein. It does not address silent mutations in the DNA or changes in the amino acid sequence that impose no structural change. RIA analysis is, in fact, measuring DNA mutation from a distance and is not as accurate as measuring DNA mutations directly.

All forms of DNA analysis are in some way measuring mutation. The value of a character resides in its ability to accurately and consistently present evolutionary variation for a given taxon. DNA is unique in its ability to present this type of information. Because DNA, barring mutagenic influence, is not an environmentally variable character, its analysis holds great taxonomic potential; however, the manner in which the genetic code may be analyzed is diverse. The methods which have been used in taxonomic studies

include karyotyping, Restriction Fragment Length Polymorphisms (RFLP), Random Amplified Polymorphic DNA (RAPD), and both organellar and genomic DNA sequencing. Only karyotyping and organellar DNA sequencing have been used to study Taxodiaceae, but the other techniques have been used for related groups and deserve consideration.

At the grossest level of DNA analysis resides karyotyping and it involves the cytological study of metaphase chromosomes. Despite the fact that this type of analysis only examines the number, sizes, shapes, and staining patterns of the chromosomes, karyotyping has been useful in resolving phylogenies. Karyotyping has been used extensively to characterize all groups of organisms and is a well established set of characters. Conducted since the beginning of the 20th century, karyotype analysis of Taxodiaceae has shown that *Sequoia* is hexaploid ($2n = 6x = 66$, allopolyploid) and provided evidence that *Sciadopitys* ($2n = 20$) is a separate family from Taxodiaceae ($2n = 22$). Karyotyping has also been useful for Taxodiaceae because of the presence of constrictions in the chromosomes called markers. This character is useful because it is not exhibited in any other family in Coniferales, except Cupressaceae. However, its usefulness for phylogenetic studies in Taxodiaceae are diminished because of inherent inaccuracies in chromosome preparation, photography, and measurement (Schlarbaum and Tsuchiya 1984). Despite these problems, karyotype analysis has certainly provided useful information for taxonomic treatments above the generic (e.g., familial, etc.).

The study of DNA sequences for taxonomic purposes began with the data collected from RFLP analysis. RFLP involves the digestion of genomic DNA into various sizes by a restriction endonuclease. The sizes of the DNA fragments are then resolved on a polyacrylamide gel, and following a Southern blot, the similarities or differences between individuals are analyzed. Because of the many restriction enzymes and radioactive probes that are available, large data sets are possible, allowing the resolution of relationships down to the species level (Song et al. 1988; Gardes et al. 1990; Gawel et al. 1992; Hosaka and

Spooner 1992). These large data sets are complex, however, and require the use of computer software such as PAUP (Phylogenetic Analysis Using Parsimony) (Swofford 1985) and PHYLIP (PHYlogeny Inference Package) (Felsenstein 1985). Although this technique has not been used to study Taxodiaceae, RFLP has been used to study the phylogeny of a wide variety of organisms (Song et al. 1988; Gardes et al. 1990; Hosaka and Spooner 1992; Gawel et al. 1992). RFLP does have disadvantages and suffers from subjectiveness. Detection of RFLP's by Southern blotting is time consuming and thus limits the number of individuals that can be studied (Williams et al. 1990), while the use of radioactive probes impose time constraints because of limited half-lives. Furthermore, the binding patterns in Southerns can can be altered by slight changes in the hybridization conditions. Despite these shortcomings, RFLP still represents a powerful taxonomic tool and should be useful in the study of genera or small families such as Taxodiaceae.

Another method of phylogenetic analysis of polymorphic DNA, RAPD, was recently developed (Williams, et al. 1990). RAPD analysis involves the detection of polymorphic DNA on an electrophoretic gel by using random sequence 9 or 10 nucleotide oligomer DNA primers to amplify segments of DNA. Unlike the typical Polymerase Chain Reaction (PCR), RAPD's use only one primer which will bind to multiple sites an both DNA strands. The amplification products are specific for each primer and are easily resolved when the gel is stained with ethidium bromide. Additionally, like RFLP, enormous data sets are possible from the 1 million possible 10 nucleotide oligomer primers. RAPD analysis has not only been useful for inter- and intra-specific phylogeny (Demeke et al. 1992; Kazan et al 1992; Vierling and Nguyen 1992; Vicario et al. 1995), but also in genetic segregation analysis of populations (Carlson et al. 1991). Unfortunately RAPD analysis seems to be restricted to the population, specific, or generic level of classification (Demeke et al. 1992; Vicario et al. 1995), so its use in studying Taxodiaceae may be limited. Studies of other conifers seems to provide evidence for this conclusion (Vicario et al. 1995). RAPD's may, however, provide useful information for the

previously mentioned disputes concerning *Taxodium* and *Athrotaxis*. Similar to RFLP's, RAPD's suffer from the subjective scoring of polymorphic bands, bands whose intensities can be affected by primer annealing conditions. Also, screening for useful primers and optimizing reaction conditions can be time consuming. Once accomplished, though, RAPD's offer the advantages of cost efficiency, ease of use, and the capability of high throughput (Williams et al. 1990).

The final extensive use of DNA for taxonomic purposes in plants involves direct sequencing of DNA. The entire DNA of an organism is, of course, too large to practically sequence for a phylogenetic study, but there are two areas of DNA that have been found to be especially useful for this type of study. One area that was originally explored for taxonomy was a region in the chloroplast genome that codes for the large subunit of ribulose-1, 5-bisphosphate carboxylase (*rbc*L). This protein is a subunit and the catalytic site of an enzyme complex that is responsible for the first step in fixing carbon dioxide in the Calvin cycle. The second area of interest involves sequencing the genes responsible for producing ribosomal RNA molecules. Because these genes encode crucial products in cellular metabolism, their sequences have remained somewhat conserved. Their conserved state is what has made these sequences attractive for phylogenetic study as well as the recovery of objective data.

The *rbc*L gene is made up of approximately 1400 nucleotides and has been used extensively for taxonomic study (Martin and Dowd 1991; Donoghue et al. 1992; Giannasi et al. 1992; Olmstead et al. 1992; Morgan and Soltis 1993; Brunsfeld et al. 1994). Recently, Brunsfeld et al. (1994) used *rbc*L sequence data to analyze Taxodiaceae and Cupressaceae. Although the earlier studies mentioned above supported using *rbc*L for intra-familial or even intra-generic studies, the data of Brunsfeld et al. (1994) indicated that the chloroplast genome evolves slower in Taxodiaceae and Cupressaceae than in the other groups studied. Consequently, *rbc*L data for Taxodiaceae were unable to fully resolve

inter-generic relationships. The *rbc*L data did support some taxonomic groupings that were in accordance with traditional morphological treatments and provided evidence for merging Taxodiaceae and Cupressaceae. Other research has used *rbc*L sequence data to look at phylogeny at the familial level or higher and found that *rbc*L data would probably need to be combined with other sequence data (e.g. ribosomal DNA) to provide meaningful results (Martin and Dowd 1991). This contrast underscores the evolutionary variability within the genomes between taxa and reinforces using preliminary studies to establish the most appropriate gene for complete sequencing.

As was mentioned earlier, in addition to *rbc*L sequencing, the ribosomal gene complex (cistron) has been useful for phylogenetic study. The strength of sequencing the ribosomal cistron resides in its size and the variability in mutation rates among its different areas. The cistron is comprised of three coding regions which encode RNA products that have sedimentation coefficients of 5.8S, 18S, and 26S and are separated by the noncoding Internally Transcribed Spacers ITS1 and ITS2. Of these five areas, the 18S and ITS1 have been the focus of most phylogenetic analysis and in other conifers they span at least 4500 nucleotides (Maggini, 1995). Although neither of these two regions have been used to characterize Taxodiaceae, they have been used for other conifer families. The 18S gene, because it represents the most conserved sequence, has been considered best suited for inter-familial relationships and higher (Nickrent 1995). However, Chaw et al. (1995) have used 18S sequences to resolve inter-generic phylogeny in conifers. In contrast to the 18S gene, the ITS1 region provides the most divergent sequence data and has been used in spruce to establish inter-specific relationships and lower (Smith and Klein 1994). Together the 18S and ITS sequence data represent a character that is flexible enough to resolve a wide variety of taxonomic levels.

Up until this point, both the object and the result of sequence data have been discussed; however, the method for resolving and detecting such data has not. Integral to sequencing a gene, though, is its amplification and this is accomplished either by cloning a

gene in a vector or with PCR. Genes with a high degree of allelic polymorphism benefit from clonal amplification because only one gene is selected; however, conserved genes such as the ribosomal cistron, are easily and accurately amplified with PCR. Once amplified, DNA may be sequenced using the dideoxyribonucleotide protocol developed by Sanger et al. (1977) or a modification of Sanger's technique called cycle sequencing. Sanger's technique utilizes the previously amplified DNA as a template for the sequencing reactions. To accomplish this, Sanger's technique incorporates a population of dideoxyribonucleotides into the reaction. The dideoxyribonucleotides cause DNA polymerization to stop when they are incorporated because they contain no 3' oxygen to facilitate elongation. To resolve and detect the various populations of termination products, four reaction tubes are set-up, each containing a different dideoxyribonucleotide (ddG, ddA, ddT, and ddC). In addition, each tube contains the DNA template, primer, buffer, sterile water, magnesium, and all four deoxyribonucleotides needed for DNA elongation. The deoxy constituents far outnumber the dideoxy representative and, as a result, elongation of the complementary DNA strand can proceed for various amounts of time before the strand-terminating dideoxyribonucleotide is incorporated. As a result, each tube has a unique population of termination products that represent the incorporation of a dideoxyribonucleotide where a deoxyribonucleotide should have been. When all four reactions are run on a single polyacrylamide gel, the termination products produce a banding pattern that may be read from the smallest fragment to the largest fragments. Depending on the lane (ddG, ddA, ddT, or ddC) the termination fragments are assigned a representative nucleotide and thus, the sequence is read.

In Sanger's original protocol, the constituents for sequencing were assembled and the reactions were allowed to incubate near room temperature to achieve the population of DNA fragments used for reading the sequence from an acrylamide gel. The resulting DNA fragments are equal in number to the templates from which they were copied. Cycle

sequencing, however, uses the thermostable *Taq* DNA polymerase in addition to the other reaction constituents and utilizes a temperature program that is similar to the ones used for PCR. Additionally, like PCR, the temperature program is repeated 30 to 60 times, but because only one primer is used in cycle sequencing, a linear amplification of DNA sequence fragments is obtained. Cycle sequencing provides higher reaction temperatures that reduce template secondary structure and offers the further advantage of amplifying the population of sequence fragments so that smaller amounts of template may be used. Also, because more DNA fragments are produced, less sensitive means of detecting the sequence may be used. The disadvantage is that if polymorphic alleles exist, one or more sequences can be produced and the gel will be unreadable.

Once the sequencing reactions have been completed and resolved on a polyacrylamide gel, the resulting data can be detected in a number of ways. The two most used methods of detecting DNA sequence bands in a polyacrylamide gel are radioactive and fluorescent, but chemiluminescence and the less sensitive silver staining may be used.

Radioactive detection of the the bands is accomplished by adding a labeled deoxyribonucleotide, usually α-[^{32}P]-dA, to the sequencing reactions. The image of the resolved bands can then be transferred to a photographic emulsion and developed. Radioactive labeling offers the advantages of sensitivity and reasonable cost, but is unfortunately hazardous and not suited for high throughput.

Fluorescence detects DNA sequence banding by attaching different colored fluorescent markers to the 4 different dideoxyribonucleotides. The reactions are carried out in different vessels as before, but may be loaded into a single lane. As the DNA fragments with end labeled dyes migrate through the gel and past a laser bank, the corresponding base is recorded. Fluorescence is sensitive, non-hazardous, stable and easily adapted for high throughput applications. Despite these advantages, fluorescent detection is prohibitively expensive because of the reagents, detection equipment, and computerware needed to score

the banding patterns.

The third detection method involves the use of a light emitting chemical to detect the DNA in the sequencing gel. To accomplish this, biotin labeled primers are used in the sequencing reaction and the resulting dideoxy termination products are resolved on a polyacrylamide gel. Once the electrophoresis is complete, the resolved DNA sequence is transferred to a nylon membrane and crosslinked to it with UV radiation. An avidin alkaline phosphatase conjugate is then bound to the DNA that contains the biotin primers. Next, a chemiluminescent substrate is added to the nylon membrane, where it is catalyzed by the bound alkaline phosphatase to an unstable intermediate that emits light as it converts to a more stable form. The image of the labeled bands can then be detected with a photographic film as the substrate emits light. Despite providing detection sensitivity comparable to radioactive and fluorescent methods, chemiluminescence has not enjoyed widespread use. Although it is safe like fluorescent labeling, the substrate is expensive and it cannot be applied to high throughput applications.

Finally, polyacrylamide gels with sufficient DNA may be stained with silver. Unlike radioactive, fluorescent, or chemiluminescent detection, sequence gels stained with silver require no labeling. The four dideoxy reactions are resolved on a polyacrylamide gel in the previously defined manner, but the band is detected by depositing silver grains onto the DNA. The image from a silver stained gel can then be easily transferred to photographic film. Silver staining is advantageous because the reagents are stable, non-hazardous, and cost-effective. Silver staining does suffer from sensitivity to contamination, higher DNA requirements, and low throughput.

RESEARCH GOALS

As discussed previously, Taxodiaceae is a family of relict genera that has resisted most attempts to resolve its phylogeny. However, with the advent of modern molecular techniques, new data are helping to characterize the family. The most advanced techniques that have been applied to Taxodiaceae (immunological analysis and *rbc*L sequencing) are in general agreement with one another and traditional morphological studies. Based on this previous work, sequencing the ribosomal 18S gene and the first internally transcribed spacer (ITS-1) is the next logical step in the study of Taxodiaceae. However, since the ribosomal genes of Taxodiaceae have not yet been characterized, a preliminary study was needed to assess their evolutionary state. The goal of this research was to provide such data by sequencing a 557 base pair DNA fragment that was amplified from the coding strand (3' end) of the 18S gene. The data were collected from the genera of Taxodiaceae that were reasonably obtainable: *Cryptomeria*, *Cunninghamia*, *Metasequoia*, *Sequoia*, *Sequoiadendron*, and *Taxodium*. A combination of cycle sequencing and silver staining were chosen for sequencing and detection because of the previously mentioned advantages of stability, cost-effectiveness, and non-hazardous nature of silver staining. Additionally, a considerable amount of time was devoted to optimizing the silver staining protocol so that ultimately, the system produced results that were accurate, reproducible, and provided a firm foundation for future research.

MATERIALS AND METHODS

Collection and Storage of Tissue. Tissue samples of the Taxodiaceae genera were collected on the campus of San Jose State University and from the private property of Dr. Rod Myatt. The *Sequoia sempervirens* and *Metasequoia glyptostroboides* tissues were collected from the southwest portion of the lawn bounded by Spartan Complex West, Spartan Memorial, and Washington Square Hall. *Sequoiadendron giganteum* samples were taken from a tree located in the middle of the lawn separating Clark Library and Dwight Bentel Hall. Within the same lawn, the *Cryptomeria japonica* samples were taken from a tree found on the west side of the Central Classroom Building. Between Dwight Bentel Hall and the Faculty Offices, the *Cunninghamia lanceolata* specimen was collected while the tissue from *Taxodium distichum* was gathered from the northeast corner of Dr. Rod Myatt's property in Gilroy, California.

All of the samples taken represented terminal branchlets from their respective plants. The samples were collected in the spring and, where possible, new growth was preferentially gathered. Terminal branchlets from the plant were removed with latex gloved hands and rinsed with distilled water. The rinsed specimens were blotted dry with KimWipes™, placed into a 5 ml cryotube, and put immediately on dry ice. Following the initial collection, the samples were placed in liquid nitrogen for storage.

DNA purification. (See Appendix A for a detailed protocol.) Tissue previously frozen in liquid nitrogen was added to an autoclaved mortar (Coors, USA II) that had been chilled on dry ice. The tissue, with some liquid nitrogen, was ground to a powder using a prechilled pestle (Coors, USA 520-1). From the ground material, approximately 100 mg of frozen tissue was used for DNA extraction. The remaining tissue was returned to the cryotube and immediately stored in liquid nitrogen.

A protocol obtained from the Forest Biotechnology Laboratory at North Carolina State University (a modification of Doyle and Doyle 1990) was used for DNA isolation from needles. Approximately 100 mg of frozen, ground tissue was transferred (with a sterilized nickel spatula) to a sterile 1.7 ml microcentrifuge tube (United Lab Plastics) containing 0.7 ml 2X CTAB extraction buffer [2.0% cetyl triammonium bromide (CTAB), 1.4 M sodium chloride, 20 mM disodium ethylenediaminetetra-acetic acid (EDTA), 1.0% PVP-10, 100 mM Tris] and 35 µl 10 mg/ml Proteinase K (Boehringer-Mannheim) which had been preheated at 65°C for 10 minutes. The tube was well mixed by inversion and incubated for 30 minutes at 65°C. Following the incubation, 0.8 ml chloroform:isoamyl alcohol (24:1) was added to the samples and inverted several times. The samples were centrifuged at 10,000 x g (Centra-M, IEC) for 5 minutes at room temperature and the aqueous phase transferred to a sterile 1.7 ml microcentrifuge tube. Again, 0.8 ml chloroform:isoamyl alcohol (24:1) was added to the aqueous sample, mixed, and centrifuged for 5 minutes at 10,000 x g. The light green aqueous phase was transferred to a sterile 1.7 ml tube and centrifuged at 10,000 x g for 5 minutes to pellet any remaining insoluble material.

Once separated from the pellet, the aqueous phase was placed into a sterile siliconized 1.7 ml microcentrifuge tube (National Scientific). One volume of -20°C isopropanol was added to the aqueous sample and gently mixed. The sample was placed in a -20°C freezer for 20 minutes to precipitate the nucleic acids, then centrifuged at room temperature for 10 minutes at 10,000 x g. The resulting supernatant was discarded and the off-white pellet was rinsed with 1 ml -20°C 70% ethanol. The pellet and wash were centrifuged at 10,00 x g for 10 minutes at room temperature and the supernatant discarded. The remaining pellet was washed with 1 ml -20°C 95% ethanol and centrifuged at room temperature for 10 minutes at 10,000 x g. The supernatant was discarded and the pellet

was dried in a vacuum desiccator (Laboratory Supply Company) for 30 minutes.

The DNA/RNA pellet was redissolved in 100 µl TE buffer [10 mM tris(hydroxymethyl)aminomethane (Tris) base and 1 mM EDTA, titrated to pH 8 with hydrochloric acid] and incubated with 2 µl 1 mg/ml DNase-free RNase A (Boehringer-Mannheim) at 4°C overnight or 37°C for 1 hour. The sample was then extracted with 100 µl chloroform:isoamyl alcohol (24:1) to remove the RNase. The sample was centrifuged at 10,000 x g for 2 minutes and the aqueous layer transferred to a sterile non-siliconized 1.7 ml microcentrifuge tube. To pellet the insoluble material, the aqueous solution was centrifuged for 10 minutes at 10,000 x g. The resulting supernatant was transferred to a sterile siliconized 1.7 ml microcentrifuge tube. One-tenth volume of 4°C 3 M sodium acetate (titrated to pH 5.5 with acetic acid) and 2 volumes -20°C 95% ethanol were added, followed by gentle mixing. The sample was allowed to sit at -20°C for 20 minutes to precipitate the DNA and then centrifuged at 10,000 x g for 10 minutes at room temperature. The supernatant was discarded and the pellet washed with 1 ml -20°C 70% ethanol, then dried under vacuum desiccation for 30 minutes. The dry pellet was redissolved in 100 µl TE buffer (pH 8) at 4°C overnight or 37°C for 30 minutes. The resulting solution was centrifuged for 2 minutes at 10,000 x g to remove any undissolved material, transferred to a sterile siliconized 0.65 ml microcentrifuge tube (National Scientific), and stored at -20°C.

DNA analysis. The presence of contaminating proteins in the isolated DNA was assessed spectrophotometrically. For the analysis, the DNA was diluted 50-fold with distilled water and placed in a 1 cm pathlength quartz cuvette. Spectrophotometer (Shimadzu, UV160U) readings taken at wavelengths of 260 nm and 280 nm were used to calculate a 260 nm/280 nm ratio. The 320 nm wavelength served as a zero absorbance reference and any deviation was subtracted from the 260 nm and 280 nm readings. Samples that had ratios of 1.8-2.0 were later used in the Polymerase Chain Reaction

(PCR). Quantitation of the DNA was based on 50 μg/ml DNA absorbing 1 unit of 260 nm light. As before, the 320 nm reading was used as a zero absorbance reference value.

The DNA was further analyzed for contaminating RNA by agarose gel electrophoresis. The samples were mixed with a 1/5 volume of 6X loading buffer (40% sucrose, 0.25% bromophenol blue, and 0.25% xylene cyanol FF) and electrophoresed (E-C Apparatus Corp., EC-135 power supply) on a 6.7 cm wide x 8.2 cm long x 0.5 cm thick 0.75% agarose gel (BioRad) in TAE buffer (2 mM Na_2EDTA, 40 mM Tris-acetate, pH 7.8) at 100 volts for 60 minutes and stained with 1 μg/ml ethidium bromide for an additional 60 minutes. A *Hin*dIII and *Eco*RI endonuclease digestion of λ DNA (Research Genetics) served as molecular weight standards. Gels were photographed on a UV transilluminator (Ultra-Lum, Midwest Scientific) with a Polaroid camera (Photodocumentation camera, Fischer Biotech; 1 second exposure, f11) using Polaroid type 667 film.

DNA amplification. (See Appendix B for a detailed protocol.) A 557 base-pair fragment of 18S ribosomal DNA was amplified using a 19 nucleotide oligomer [NS1-d(GTAGTCATATGCTTGTCTC)] and 21 nucleotide oligomer [NS2-d(GGCTGCTGGC-ACCAGACTTGC)] primer (White 1990). The PCR samples were prepared in a 0.5 ml PCR tube (Perkin-Elmer) in a total volume of 25 μl. Included in the sample were 14.75 μl of sterile Millipore water, 3.0 μl of 25 μM magnesium chloride (Perkin-Elmer), 2.5 μl of 10X Stoffel buffer (Perkin-Elmer), 2.0 μl of 2.5 mM (each) deoxynucleotide triphosphates (dNTP's, Boehringer-Mannheim), 0.5 μl each of 20 μM NS1 and NS2 primers (Research Genetics), and 0.25 μl of 10 U/μl *Thermus aquaticus* DNA polymerase-Stoffel fragment (Perkin-Elmer). After the reactants had been assembled on ice in the PCR tube, 1.5 μl of 20 ng/μl sample DNA were added and gently mixed. The reaction vessel was then placed in a Peltier effect thermocycler (MJ Research, PTC-100™ with Hot Bonnet™ heated lid) and its contents denatured for 1 minute at 95°C. Following the initial denaturation, the

cycle program began. The first step was a 1 minute 95°C denaturation and was followed by a 1 minute 55°C primer annealing phase. The next step, 72°C for 2 minutes, allowed the PCR fragments to elongate. The temperature program repeated 29 times and was followed by a final 5 minute 72°C step.

The PCR product was separated from the nucleotides and primers by combining the volumes of triplicate reactions and applying them to a gel filtration spin column (5 Prime→ 3 Prime, PCR Select® III Spin Columns). Prior to the sample application, the gel was resuspended in the column and allowed to settle in the upright position. Once settled, the caps on the column were removed and the column was placed in a collection tube. The tube and column were then centrifuged in a swinging bucket rotor (IEC, model 218) at 1000 x g for 1 minute at room temperature. The eluted buffer was discarded, and the column and collection tube were centrifuged at 1000 x g for 5 minutes. Following centrifugation, the collection tube and residual buffer were discarded and a new tube connected to the column. Approximately 75 µl of PCR product was applied to the top of the gel. Special care was taken to keep the sample from touching the column sides. The column was then centrifuged for 3 minutes at 1000 x g. The resulting column eluate was then assessed for PCR product purity by mixing 5 µl of the sample with 1 µl 6X loading buffer and electrophoresing on a 0.75% agarose gel. To quantify the PCR product, the sample was diluted 100-fold with distilled water and analyzed spectrophotometrically.

DNA cycle sequencing. (See Appendix C and D for a detailed protocol.) PCR products were sequenced using the DNA SILVER SEQUENCE kit from Promega. Four 0.5 ml PCR tubes (Perkin-Elmer) labeled G, A, T, and C were filled with 2 µl of their respective deoxy/dideoxyribonucleotide triphosphates (d/ddNTP, Promega) (ddG is substituted with 7-deaza-2'-deoxyguanosine triphosphate to reduce potential secondary structure), overlaid with 10 µl of mineral oil (US Biochemical), and placed on ice. In a

separate 0.65 ml PCR tube, 4.75 µl of sterile Millipore water, 5.0 µl 5X DNA sequencing buffer (Promega), 4.5 pmol PCR primer (NS1 or NS2), and 120 fmol PCR template were added in their respective order. Finally, 1.0 µl of 5 U/µl *Taq* DNA polymerase (Promega) was added and the tube well mixed. From the 17 µl mixture, 4 µl were placed on the inside wall of each of the tubes previously labeled G, A, T, and C and the reaction mixes were centrifuged at 10,000 x g for 10 seconds. The bottom of each tube was dipped in mineral oil and placed in the thermocycler equipped with the heated lid.

If the primer had fewer than 24 bases or the GC-content was less than 50% (i.e., NS-1), the cycle sequence reaction had an initial 2 minute 95°C denaturation followed by a 30 second 95°C denaturation step, a 30 second 42°C annealing step, and a 1 minute 70°C extension step. The temperature program would then cycle 60 times. However, if the primer had 24 or more bases or the GC-content was greater than or equal to 50% (i.e., NS-2), another temperature program was needed. Following the initial 2 minute 95°C denaturation, the samples were cycled 60 times through a 30 second 95°C denaturation step and a 30 second 70°C annealing/extension step. Following the completion of all programs, the samples were held at 4°C until 3.0 µl of DNA sequence stop solution (Promega) could be added. The samples were then mixed, centrifuged for 10 seconds at 10,000 x g and placed at -20°C for storage.

The sequencing products were resolved on a Jordan Scientific sequencing apparatus (PhorRunner™) that measured 18.5 cm x 42 cm and 18.5 cm x 62 cm for the regular and extended gels, respectively. Each gel preparation consisted of a set of 2 glass plates that differed in length by 1.5 cm and were referred to as either short or long. The glass plates for the sequencing gel were cleaned using gloved hands to apply Alconox® and distilled water with a synthetic, non-abrasive sponge. The cleaned plates were rinsed with distilled water, 95% ethanol and then allowed to dry in an upright position.

The short plate was treated with Bind Silane™ (Sigma) to promote adherence of the polyacrylamide gel. The preparation involved the addition of 3 µl Bind Silane™ to 1 ml of 0.5% glacial acetic acid in 95% ethanol. The solution was mixed and approximately 500 µl was applied to one side of the short glass plate with a KimWipe™ using parallel strokes over the length of the plate. If the KimWipe was still wet, an additional pass, perpendicular to the first, was performed. Starting from the opposite end of the initial application, the remainder of the binding solution was applied to the glass plate and wiped as described above. The Bind Silane™ was allowed to sit for 5 minutes. Excess reagent was removed with a new KimWipe™ wetted with 95% ethanol using medium pressured parallel strokes over the entire surface. This was repeated 3-4 times with each successive step performed perpendicular to the previous one, until the glass was free of any haze.

The long plate was prepared with the hydrophobic releasing agent Acrylease™ (Stratagene). Prior to the application of Acrylease, however, latex gloves were changed. A KimWipe™ was then wetted with two full pumps from the spray bottle and, as before, the reagent was applied in parallel strokes over the entire surface and then again in a perpendicular direction. Following the Acrylease™ application, a KimWipe™ lightly dampened with distilled water was rubbed over the long plate. Preparation was completed when a dry KimWipe™ was used to buff the entire surface.

The prepared plates were assembled by placing the 0.44 mm thick spacers along the left and right sides of the long plate. The short plate was then placed on top of the spacers and held along one edge with 4 clips (VWR, CBG PC-0002). The glass plates were assembled with the prepared surfaces facing each other. The plates were then pried apart 1 cm along the non-clipped edge and compressed air was blown between them. A 1 ml plastic pipet tip with 1 cm of the tip removed was used to direct the stream of air. The air treatment between the plates was continued until all visible dust had been removed. Once the plates had been meticulously cleaned, the spacer along the free edge was realigned and

held in place with clips at the top and bottom of the plates. Gel casting tape (Permacel) was applied to the edge of the plates between the two clips and smoothed to exclude any air bubbles. Once completed, clips were placed along the entire edge of the newly taped side. The continuous piece of tape was then extended along the bottom and secured before moving up the other side. The bottom was left unclipped because it lacked a spacer and was simply present as a barrier to the gel. Clipping the bottom also leads to gel thickness variation and staining anomalies. To continue taping up the other side, all but the top clip was removed. When the taping was complete, the clips were replaced along the edge. One final piece of tape was placed along the bottom of the plates in case the original layer had been pierced. Once taped and clipped, the plate structure was ready for the acrylamide gel. An 8% acrylamide solution with 5% crosslinking was prepared by dissolving dry ultrapure DNA sequencing gel mix (US Biochemical, #70090) in 75 ml millipore water. From this, a 6% acrylamide solution was prepared by the addition of 15.4 g urea (BRL), 3.67 ml 10X TBE buffer (0.89 M Tris base, 0.89 M boric acid, 20 mM EDTA at pH 8.0), and Millipore water to a final volume of 160 ml in a 250 ml graduated cylinder. The solution was stirred under a vacuum with a magnetic stir bar in a 250 ml Erhlenmeyer flask. Once dissolved, the 6% acrylamide was poured into an open 60 ml syringe equipped with a syringe filter apparatus (Millipore, Swinnex-25) that contained a 2.4 cm glass microfiber filter (GF/A, Whatman) that had been previously flushed with 20 ml 1X TBE buffer. The filtered acrylamide solution was placed back in a vacuum for 5 minutes, covered with Parafilm®, and stored at 4°C for up to 2 weeks.

The polymerized acrylamide gel was prepared by withdrawing 40 ml (small gel) or 60 ml (large gel) of the 6% acrylamide stock solution with a 60 ml syringe. Through the tip of the syringe, 405 µl of fresh 10% (w/v) ammonium persulfate (Bio-Rad) and 10 µl TEMED (Bio-Rad) were added to the acrylamide and gently mixed. A 1 ml plastic pipet tip

was placed on the end of the syringe and any air trapped in the syringe was carefully expelled. The tip of the pipet was placed in the upper right corner of the prepared plates as they were tilted 45° back and slightly towards the syringe. The acrylamide was slowly added until it had filled 75% of the space between the plates. The remaining space filled as the plates were laid flat. An excess bead of acrylamide was placed along the top of the plates and the flat side of the Picket Fence comb was inserted 6 mm into the gel. The gel was allowed to polymerize for one hour before the tape along the bottom and the clips along the sides were removed.

The polymerized gel was placed into the sequencing apparatus and lightly clamped into position. Once the upper buffer reservoir was clamped, both buffer tanks were filled with 250 ml 1X TBE buffer. When the buffer was added to the top reservoir, the comb was removed and the resulting space was rinsed with tank buffer using a 1 ml pipetter. The polyacrylamide gel was then pre-electrophoresed for 30 minutes at 55 W using a Fischer (FB 500) power supply. Excess urea that had diffused from the gel during the pre-electrophoresis was flushed from the well space with the 1 ml pipetter and the comb was inserted pickets first. The tips of the comb were placed so that they lightly touched the gel. Next, 125 ml unbuffered 3M Sodium Acetate was added to the 250 ml 1X TBE in the lower buffer tank. The upper buffer tank was diluted to 0.5X TBE by the addition of 250 ml distilled water. The sequencing products were then heated for 2 minutes at 70°C and 3 µl of each sample were placed into the wells formed by the comb with a special loading tip (Multiflex Microcapillary pipet tips, 0.37 mm flat tip, PGC Scientifics Corp.). The sequencing gel was electrophoresed at 34 Watts for 50 to 300 minutes. Once electrophoresis was completed, the gel plate was removed from the sequencing stand and placed on a flat surface with the short plate down. The tape and one of the spacers were removed and the plates pried apart with a plastic wedge. The sequencing gel, adherent to

the short plate, was then ready to be fixed, stained, and developed.

The short plate (with the polyacrylamide gel) was placed in a 28 quart Rubbermaid® container (JL3-2221) for 30 minutes with a fix/stop solution (10% acetic acid) that consisted of 200 ml glacial acetic acid (Baker) that had been diluted with 1.8 L Millipore water. While the gel was fixing on a shaker at 75 rpm, the developing solution was made by dissolving 60 g of high quality sodium carbonate (Fischer, #S263-500 or Promega, included with the Silver Stain) in 2 L of rapidly stirring Millipore water. Once the sodium carbonate had dissolved, the solution and a 28 quart Rubbermaid® container were placed in the -20°C freezer (solution is 4°C-10°C by the time it is used later). Following fixation, the acetic acid bath was saved and the gel was rinsed 3 times. Each was shaken at 75 rpm for 2 minutes in 2 L Millipore water that was well drained from the gel before transferring it to the successive rinse. After the final rinse, the gel was transferred to the staining solution that contained 2 g silver nitrate, 3 ml 37% formaldehyde, and 2 L Millipore water and shaken as before. The gel was stained for 30 minutes and immediately rinsed with 2 L Millipore water. The wash lasted only 15 seconds from the time the gel touched the water until it was immersed in the developing solution and was not shaken. However, before the developing solution (chilled to 4°C-10°C) was poured into the prechilled container, it was mixed with 3 ml 37% formaldehyde and 0.4 ml 10 mg/ml sodium thiosulfate. The gel was developed for 8-10 minutes while shaking at 75 rpm and stopped by the addition of 2 L recovered 10% acetic acid. The gel remained in the stop bath for 3 minutes and was rinsed twice with 2 L Millipore water for 2 minutes each. Following the final rinse, the glass plate and adherent gel was placed flat to dry. A cover was made from the Rubbermaid® containers so that dust would not settle on the plates and yet air flow was not restricted.

Once the gel had dried on the glass plate, the DNA sequence was transferred onto APC film (Promega, Q4411). The image on the sequencing gel was transferred by taping

the glass plate to a white fluorescent lightbox (Picker International, #240096) so the gel was facing up (away) from the light source. Under an orange safelight in a darkroom, the film was placed with the white, shiny side down and covered with another glass plate. Using a construction paper shutter between the light source and the glass/gel plate, the lightbox was turned on with the shutter in place. The paper shutter was then removed and the film was exposed to fluorescent light for approximately 6.5 seconds. Because of variation in the amount of background staining exposure time varied ±1 second. Once the film had been exposed, it was placed in Kodak® GBX Developer for 1 minute followed by a 10 second distilled water wash. Once washed, the film was placed in Kodak® GBX Fixer for 4 minutes. The fixed film was then transferred to a distilled water wash for 10 minutes and hung to dry. The dry film, with the reverse image of the DNA sequencing ladders, was then labeled and either read with or without the lightbox.

RESULTS

DNA purification. DNA isolated from various Taxodiaceae samples with a protocol modified from Doyle and Doyle (1987) produced a good yield of high molecular weight DNA that had no RNA contamination as judged by ethidium bromide staining of the gels (Figure 1). Yields for the six genera were quantified by spectrophotometry and readings taken at an absorbance of 260 nm indicated that DNA recovery differed nearly two-fold from 11.75 μg to 21.55 μg for the 100 μl samples (Table 1). The difference in DNA yield was attributed to variation in the amount of starting tissue and not inherent differences between the samples. Further spectral analysis indicated that the DNA samples had insignificant protein contamination. Calculated 260 nm/280 nm absorbance ratios of 1.8 to 2.0 were considered protein free and sample values ranged from 1.85 to 1.95 (Table 2).

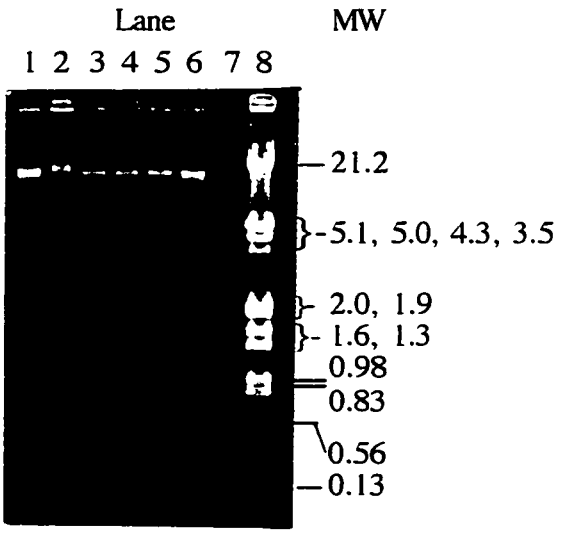


Figure 1. Ethidium bromide-stained 0.75% agarose gel with DNA isolation products. Lane 1 = *Taxodium;* Lane 2 = *Cryptomeria;* Lane 3 = *Cunninghamia;* Lane 4 = *Metasequoia;* Lane 5 = *Sequoiadendron;* Lane 6 = *Sequoia;* Lane 8 = λ molecular weight marker (*Hin*dIII and *Eco*RI digested).

TABLE 1

Genus and 260 nm DNA quantitation

Genus	260 reading*	DNA conc.	DNA yield
Sequoia	0.085	212.5 µg/ml	21.25 µg
Sequoiadendron	0.047	117.5 µg/ml	11.75 µg
Metasequoia	0.084	210.0 µg/ml	21.00 µg
Cunninghamia	0.072	180.0 µg/ml	18.00 µg
Cryptomeria	0.052	130.0 µg/ml	13.00 µg
Taxodium	0.074	185.0 µg/ml	18.50 µg

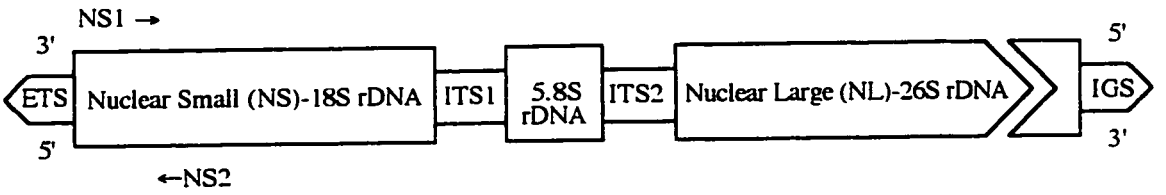
* DNA has been diluted 50-fold

TABLE 2

Genus and DNA purity measured by 260/280 ratio

Genus	260/280 readings	Ratio
Sequoia	0.085/0.046	1.85
Sequoiadendron	0.047/0.025	1.88
Metasequoia	0.084/0.045	1.87
Cunninghamia	0.072/0.037	1.95
Cryptomeria	0.052/0.028	1.86
Taxodium	0.074/0.038	1.95

DNA Amplification. Purified genomic DNA was amplified with PCR using primers NS1-d(GTAGTCATATGCTTGTCTC) and NS2-d(GGCTGCTGGCACCAGACTTGC) (White 1990). These primers amplify a 557 nucleotide segment of 18S rDNA that is at the 3' end of the coding strand in the ribosomal cistron (Figure 2).



Ribosomal Gene Complex

Figure 2. Diagram representing the ribosomal genes and the section of DNA that was amplified by primers NS1 and NS2, a 557 nucleotide area. ETS is the External Transcribed Spacer. ITS is the Internal Transcribed Spacer 1 and 2. IGS is the InterGenic Spacer that spans the region between the 26s rDNA gene and the ETS region among tandem repeats (White 1990; Smith and Klein 1994).

Results of the PCR (Figure 3) showed a major amplification product of the expected size for each genus. The diffusely stained area at the bottom of each lane include unincorporated primers, nucleotides, and incomplete amplification products. A control reaction, without a DNA template, yielded no amplification product , but did present a diffusely stained area at the bottom of the gel (not shown). Successful amplification reactions were combined into a single volume to facilitate purification with a spin column. The effectiveness of the spin column at removing nucleotides, primers, and incomplete PCR products which would interfere with the subsequent sequencing reactions is evident in Figure 4. The PCR products were quantified using a pool of the amplified DNAs because uniform yields, as judged by ethidium bromide staining, were obtained. To the 30 µl DNA pool, each genus contributed 5 µl. The resulting DNAs were diluted 30-fold and analyzed spectrophotometrically at 260 nm as described for the DNA purification. The estimated

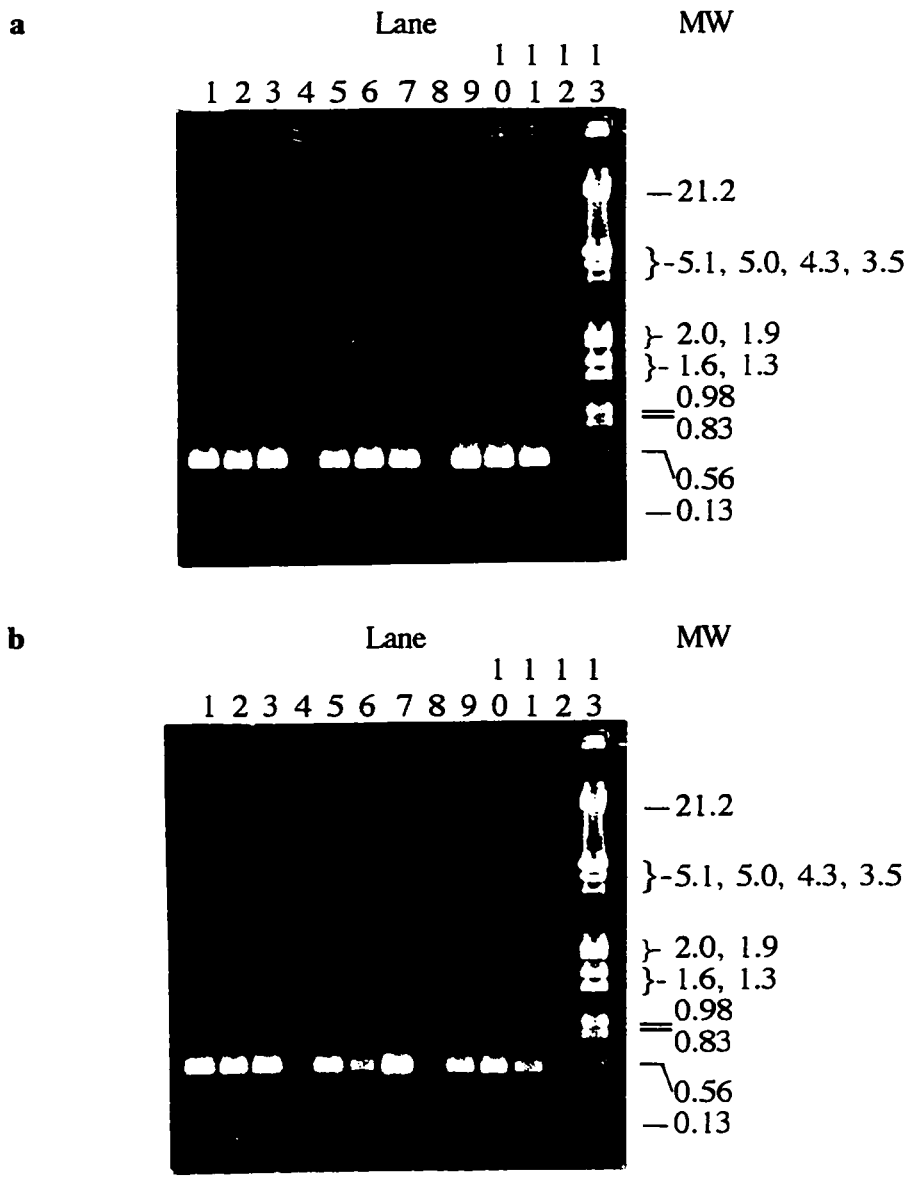


Figure 3. Both figures show 5 µl of PCR products (from triplicate 25 µl reactions) from genera in the Taxodiaceae family. Products were separated on a 0.75% agarose TAE gel and subsequently stained with ethidium bromide. **3a** represents the PCR products from *Metasequoia*-lanes 1-3, *Sequoiadendron*-lanes 5-7, and *Sequoia*-lanes 9-11, with a λ molecular marker (*Hin*dIII and *Eco*RI digested) in lane 13. **3b** represents *Taxodium*-lanes 1-3, *Cryptomeria*-lanes 5-7, and *Cunninghamia*-lanes 9-11, with the same λ molecular marker in lane 13. Lanes 4, 8, and 12 in each figure are empty.

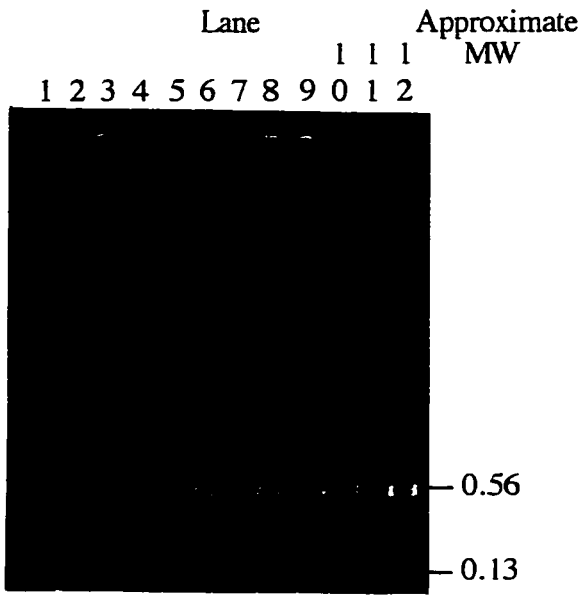


Figure 4. Ethidium bromide-stained agarose gel demonstrating the effectiveness of the spin columns at removing low molecular weight primers, nucleotides and incomplete amplification products from the PCR reaction of the six genera. *Taxodium*-centrifuged with (lane 1) and without (lane 2), *Cryptomeria*-centrifuged with (lane 3) and without (lane 4), *Cunninghamia*-centrifuged with (lane 5) and without (lane 6), *Metasequoia*-centrifuged with (lane 7) and without (lane 8), *Sequoiadendron*-centrifuged with (lane 9) and without (lane 10), *Sequoia*-centrifuged with (lane 11) and without (lane 12).

DNA concentration (60 µg/ml) was then used for calculations in setting up the cycle sequencing reactions. Figures 3a and 3b demonstrate the relatively uniform quantities of DNA produced by PCR.

DNA sequencing. Early attempts at cycle sequencing and silver staining were met with widespread failure. As a result, a great deal of time was spent identifying errors and optimizing the silver stain protocol. Indeed, the entire process for silver staining was exquisitely sensitive to minor procedural deviations. However, most errors occurred in the preparation of the sequencing glass plates with only a few in electrophoresis and staining.

Plate preparation and subsequent pouring of polyacrylamide were the most important parts of the silver staining process and mistakes would ultimately lead to uneven migration, staining aberrations, and poor plate separation.

High background and low contrast were caused by glass plates which had a detergent residue left on them. Cleaning agents such as Microclean did not work well and the resulting gels had high staining background. Excessive background was eliminated when the glass plates were cleaned with Alconox®, rinsed with distilled water and followed with a 95% ethanol rinse.

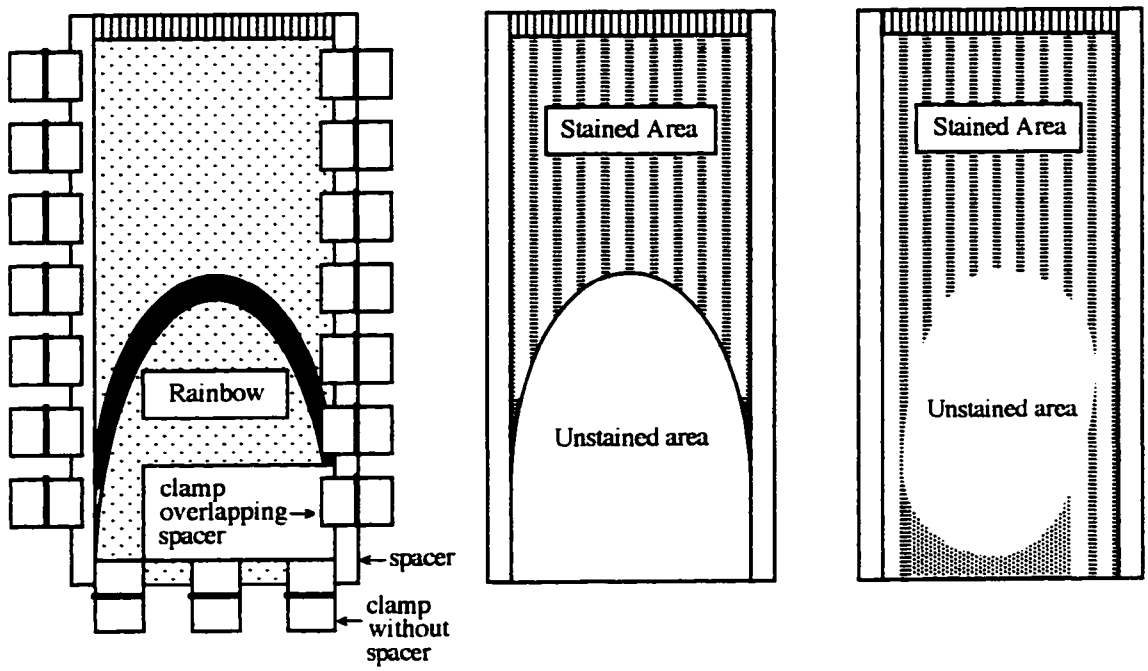
Improper preparation of the cleaned plates brought a host of new problems. To support the 0.4 mm thick polyacrylamide gel during electrophoresis and make it available for staining, the gel must bind to one plate and yet must release easily from the other. The short plate must be treated with Bind Silane™ which covalently binds the polyacrylamide gel to the glass thereby allowing subsequent agitation during the staining procedure. However, excess Bind Silane™ and slowly polymerizing polyacrylamide were suspected of allowing the binding agent to diffuse across the gel and crosslink it to both plates. The slow polymerization was probably caused by oxygen contaminated acrylamide solutions. Tearing stopped once the polyacrylamide was put under vacuum prior to polymerization (acrylamide solution was not over 2 weeks old) and excess Bind Silane™ was thoroughly removed from the glass plate. For easy removal of the long glass plate from the gel, the

plate must be treated with a hydrophobic "releasing" agent such as Sigmacote™ or Acrylease™. Excess releasing agent did not apparently effect gel tearing, but did reduce the DNA banding intensity on the stained gels. This became apparent when well-stained, yet torn gels, were compared to gels that had been well lubricated with releasing agent. Thorough removal of Sigmacote and the eventual switch to the releasing agent Acrylease, restored banding intensity.

Poor resolution was also a problem with sequencing gels because there was particulate matter (dust) in the gels that attracted the positively charged silver and occluded the DNA banding. The dust was eliminated and resolution improved when the polyacrylamide was filtered and the glass plates were blown free of dust with compressed air.

Polyacrylamide gel pouring and polymerization was the most troublesome aspect of DNA sequencing. Uneven gel staining and migrating DNA were caused by mistakes made while clamping the glass plates together with their spacers. Misplaced clamps led to tracking dyes that formed a "rainbow" during electrophoresis (Figure 5a) and then left a large unstained area once the silver staining was complete (Figure 5b). It was determined that clamps placed along the bottom of the plates, where no spacer existed, were bending the glass and compressing the gel. It was also determined that if the clamps along the edge were allowed to overlap the spacer, they too could compress the gel (Figure 5a). Once the clamps were properly positioned, another problem appeared. All gels had been polymerized in an upright position and the clamping problems had masked the fact that the weight of the gel caused the glass plates to bulge at the bottom. The resulting gel caused the tracking dyes to form a "smile", while staining left an area that was clear in the lower portion of the gel (Figure 5c). Proper placement of the clamps on only the spacers and allowing the polyacrylamide to polymerize while laying flat resolved all the uneven staining and mistracking dye problems.

Another source of poor band development arose during electrophoresis and was the



a. Correct clamp placement along the left side. Incorrect clamp placement along the bottom and right side.

b. Staining results when clamps overlap spacers or the bottom is clamped without them.

c. Staining results when clamps are positioned properly, but the gel is polymerized upright.

Figure 5. Diagrams represent potential problems with polyacrylamide gels. The diagram at the left shows the proper and improper clamping positions as well as the way an improperly clamped gel would look as it was electrophoresed. The center diagram shows what an improperly clamped gel would look like stained. The right diagram represents a stained gel that had been clamped properly, but allowed to polymerize in an upright position.

result of an overheated sequencing gel. The ideal temperature range is 40-50°C and excess heat can degrade the polyacrylamide, causing faint banding at the top of the gel with normal banding at the bottom. Although it is important to maintain a minimum gel temperature to reduce secondary structure of the sequencing products, too much heat leads to reduced band intensity and reduces the length of readable sequence. Running the gel at 32-34 Watts provided the best results for denaturing secondary structure and not degrading the polyacrylamide gel.

The final optimization was made in Promega's Silver Staining protocol and produced sequencing gels that were more evenly stained, had less background and improved resolution above and beyond the previously mentioned considerations. Despite the fact that the unstained areas resolved with proper clamping, the staining was still not homogeneous and subject to darker staining bands running through the gel. After examination, the darker areas revealed they were sites of excess silver that had not been properly rinsed. Apparently the 10 second wash proved to be too short and left residual silver on the glass plate. As a result, the excess silver would precipitate and stick to the gel, causing an uneven stain. Although the protocol warns against it, the rinse step was lengthened to 15 seconds and the resulting gels produced even staining.

The use of the proper cleaning reagents helped reduce most of the background staining, but the quality of the final stain was also affected by the temperature of the developing solution. A high background stain could also result from excessively warm (greater than 10°C) developing solutions. Development of the gel in a pre-chilled tray helped to maintain temperature and the appearance of the background was more controllable

Once the gel had been stained and dried, the image was transferred to APC film. The stained sequencing gel was placed on top of a light box and the APC film was then placed on top of the gel. Unfortunately, the transferred images were often of poorer quality than the original and unevenly developed because the two fluorescent tubes in the lightbox

rarely lit simultaneously. The improved resolution from filtering the unpolymerized gel solution and the staining improvements were lost in the image transfer. However, the images were improved by placing a black construction paper shutter between the lightbox and the sequencing gel. Both lights could then turn on before the film was exposed by removing the shutter. As for the poor resolution, it disappeared when the minute air space between the gel and film was removed by the placement of a glass plate on top of them.

Once the problems with the methodology had been corrected, Promega's silver sequence process was able to consistently produce sequencing gels that provided proper DNA ladder development, consistent staining, and appropriate contrast. The protocol provided up to 250 nucleotides in readable sequence per run (Figure 6). Using the forward and reverse primers from the PCR, nearly all of the 557 base PCR product was readable and is available in Figure 6. However, despite increasing the annealing temperature and using formamide polyacrylamide gels to minimize secondary structure, an artifact of unknown origin occupied the lanes for the first forty bases after each primer. The artifact, multi-lane ladder development for each sample, was not found in the positive control and was not equal in its intensity for all six genera studied.

The sequence itself, however, was the same for all the genera with the exception of one transition (G→A in the coding strand) in *Metasequoia* at position 190 (Figures 6 and 7). The sequence listed in Figure 7 represents the transcript of the coding strand according to literature convention. The sequence, therefore, depicts the DNA 5'→3' with the NS-1 primer incorporated into the sequence 20 bases downstream from the beginning of the ribosomal 18S gene. The 3' end of the sequence identifies the annealing sequence for the NS-2 primer.

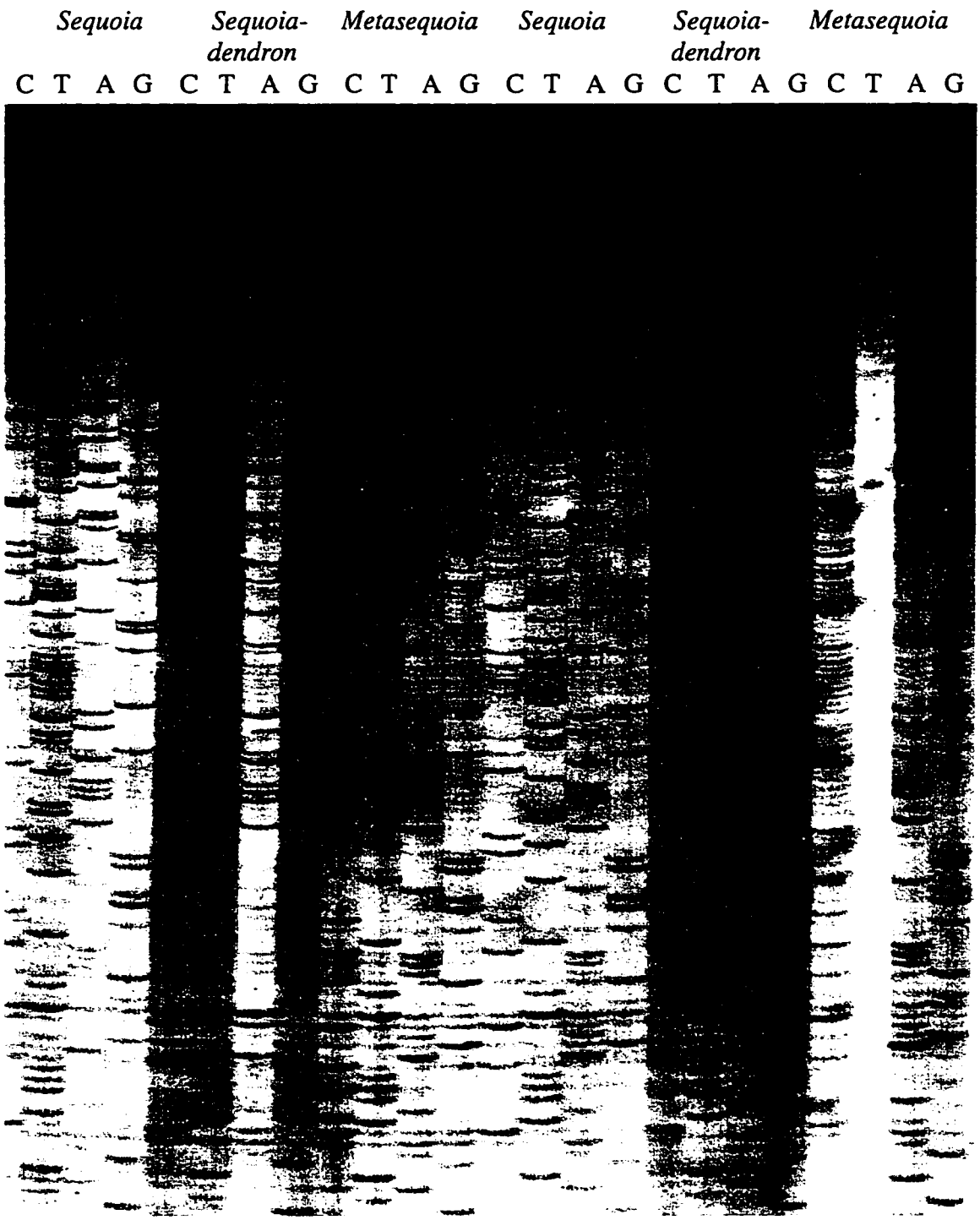


Figure 6. Representative image of DNA sequence ladders resolved on a 6% polyacrylamide gel for 75 minutes and detected with silver stain. The sequence ladders were produced with cycle sequencing using the NS-1 primer for *Sequoia, Sequoiadendron,* and *Metasequoia.* The arrow indicates the single mutation that was detected.

	1	11	21
Sequoia sempervirens (Ss)	5'-NNNNNNNNNN	NNNNNNNNNG	TAGTCATATG
Sequoiadendron giganteum (Sg)			
Metasequoia glyptostroboides (Mg)			
Cunninghamia lanceolata (Cl)			
Cryptomeria japonica (Cj)			
Taxodium distichum (Td)			

	31	41	51	61
Ss	CTTGTCTCNN	NGNTTAAGNN	ATGCATGTCT	AAGTATGAAC
Sg		N NNNNN	N	
Mg			N	
Cl		N NN CC		
Cj		N NNNNN		
Td		N NN CC		

	71	81	91	101
Ss	TATTTCAGAC	TGTGAAACTG	CGGATGGCTC	ATTAAATCAG
Sg				
Mg				
Cl	N	NN		
Cj	N			
Td				

	111	121	131	141
	TTATAGTTTC	TTTGATGGTA	CTTTGCTACT	CGGATAACCG

	151	161	171	181
	TAGTAATTCT	AGAGCTAATA	CGTGCACCAA	ATCCCGACTC

Figure 7. Sequence for the 557 nucleotide PCR product amplified from the 18s ribosomal gene. The sequence depicted represents the coding strand transcript from *Sequoia sempervirens*. There were no variations from the *Sequoia* sequence from bases 83-513 so only the *Sequoia* sequence is shown. Any variation from the *Sequoia* sequence by the other genera is indicated immediately below the base. Variation, however, does not indicate mutation, but various levels of sequence readability. Where the sequence could not be unambiguously determined, an N appears. Dashed sequences represent the primer (NS-1, bases 20-38) or its binding site (NS-2, bases 555-576). Outlined sequence (bases 223-225) indicates the positions where *Cunninghamia* could not be read. The underlined base (position 190) shows the only mutation that was detected among the six genera; *Metasequoia* underwent a transition mutation (G→A in the coding strand).

	191 TTGGAAGGGA	201 TGCATTTATT	211 AGATAAAAGG	221 CCGGCGCGGG
	231 CTCGCCCGCT	241 ACTCCGGTGA	251 TTCATGATAA	261 CTCGACGGAT
	271 CGCACGGCCT	281 TTGTGCCGGC	291 GACGCTTCAT	301 TCAAATTTCT
	311 GCCCTATCAA	321 CTTTCGATGG	331 TAGGATAGAG	341 GCCTACCATG
	351 GTGGTGACGG	361 GTGACGGAGA	371 ATTAGGGTTC	381 GATTCCGGAG
	391 AGGGAGCCTG	401 AGAAACGGCT	411 ACCACATCCA	421 AGGAAGGCAG
	431 CAGGCGCGCA	441 AATTACCCAA	451 TCCTGACACG	461 GGGAGGTAGT
	471 GACAATAAAT	481 AACAATACTG	491 GGCTCATCGA	501 GTCTGGTAAT
Ss Sg Mg Cl Cj Td	511 TGGAATGAGT N NN NNNN	521 ACAATCTAAA NNNNNNNNNN	531 TCCCTTAACG NNNNNNNNNN	541 AGGATCCATT NNNNNNNNNN NNNNNNNNNN
Ss Sg Mg Cl Cj Td	551 GNNNNGCAAG N N N	561 TCTGGTGCCA 	571 GCAGCC-3' 	

Figure 7. Continued.

DISCUSSION

Silver staining proved to be a viable system for detecting DNA sequence data from PCR products generated from the 18S rDNA of the six Taxodiaceae genera studied. Although a great deal of time was spent optimizing the sequencing protocol, only a few of the problem steps were unique to the Silver Sequence methodology. Those that were unique were isolated to the staining process, a process not shared with other sequence detection methods. Problems with detergent residues and developer preparation led to poorly contrasted gels with high background staining while dust and inadequate rinsing after the staining produced uneven silver deposition. The remaining problems encountered with silver sequence had common aspects to other sequencing methodologies.

Proper preparation of an acrylamide gel is certainly an important facet of most sequencing. The compressions and bulges encountered in the present study would have certainly been noticed with any detection method through mis-migrating bands, silver staining just highlighted the variations in the gel thickness. Another commonality was the prevalence of tearing gels. Since glass plate separation and subsequent drying are found in many protocols, information acquired with silver staining about the proper use of binding and release agents with fresh, oxygen-free acrylamide has widespread applications. Additionally, many DNA detection systems such as isotopic or chemiluminescence, rely on transferring images to film to preserve and even read the sequence. Any of these techniques would certainly benefit, as silver staining did, from removing air from between the film and sequence to create as close a connection as possible. Certainly, many of the problems encountered with the Silver Sequence methodology stemmed from unfamiliarity with sequencing systems in general. Personal communications have indicated that many believe Silver Sequencing to be problematic from personal experience or hearsay. Such experience may reflect the allure of silver sequencing, because of its safety and cost-

effectiveness, to novice sequencers.

Low cost is certainly an attractive aspect of Silver Sequencing. On one level, silver staining does not require the disposal of radioactive material, not to mention the potential health benefits gained from not working with radioisotopes. On the other level, compared to other non-radioactive detection methods such as chemiluminescence and fluorescence, silver staining is not exorbitantly priced. In addition, the reagents and enzymes in the Silver Sequence kits provided for cycle sequencing and silver staining were very stable. The Silver Sequence reagents were effective up to three years (data not shown), an important aspect for low throughput labs or where teaching applications might be considered. Silver Sequencing provides a safe, cost-effective means to effectively sequence DNA.

However, unlike the other detection techniques that clonally amplify single alleles, Silver Sequencing's detection method is less sensitive and requires cycle sequencing to amplify the alleles. Despite the fact that cycle sequencing offers the advantage of limiting secondary structure formation with high reaction temperatures, its link to silver staining limits the applications of the Silver Sequencing system. Because any gene that binds the primer is sequenced, cycle sequencing is unsuitable for polymorphic genes and will produced unreadable overlapping sequences when resolved on a polyacrylamide gel. Cycle sequencing is still useful for alleles with high homology such as the ribosomal genes and produces sequence accuracy that is equivalent to that provided from Sequenase®, the most commonly used sequencing system (Koop et al. 1993). Indeed, the results from the present study confirm the accuracy of cycle sequencing if the sequence for the six genera are compared; a span of approximately 3000 bases. Besides displaying the accuracy of the cycle sequencing, the sequences produced from the six Taxodiaceae genera displayed a high degree of conservatism and were nearly all identical, only one transition was found in *Metasequoia*. The identical sequences and the lone mutation are certainly striking

characters that raise questions as to the validity of the data.

The sequences were derived from PCR products and the possibility exist that a single ubiquitous contaminate was the source of the amplification and subsequent identical sequences. However, a PCR control that contained no DNA, was unable to produce banding on an agarose gels and, yet, showed the characteristic diffuse staining at the base of the gel that represented the primers and nucleotides (data not shown). Although contamination from a reagent source was excluded, tissue contamination was not, but due to the collection of the samples from a campus wide distribution and one from many miles away, such contamination was probably not likely.

The single *Metasequoia* mutation also needs to be addressed because cycle sequencing is known for producing consistent false mutations (Koop et al. 1993). Despite such a seemingly serious flaw, such mutations are unidirectional and cycle sequencing is capable of double checking the sequence from the reverse direction. When the complementary 18S strand of *Metasequoia* was sequenced, the mutation was confirmed.

Further support for the sequence homology can also be found in the data gathered from related families. In fact, when the sequences of the six genera are compared to the Pinaceae family, only two mutations are observed (Chaw 1993). The conclusion, therefore, is that the sequences determined are accurate representations of the 18S rDNA genes in the six genera of Taxodiaceae.

In addition to the homologous readable data (i.e., sequence) there were numerous artifacts shared by most genera in the sequences closest to the primers. The artifacts produced areas within the DNA ladder that were unreadable because of multiple lane banding (see Results, Figures 6 and 7). Many attempts were made to resolve the problem and it was believed to be secondary structure occurring either within the gel or in the template during the sequencing reactions. The formation of secondary DNA structures within the gel was ruled out for a number of reasons. First, the sequencing reactions utilize

7-deaza-2'-deoxyguanosine triphosphate, a nucleotide analog that precludes base stacking and prevents secondary structure (Innis 1990). Also, secondary structure has been known to arise from low polyacrylamide running temperatures (Promega 1995), but this reason was dismissed because the gels in the present study were adequately warm, sometimes overly so, resulting in partially degraded polyacrylamide. Finally, a polyacrylamide gel with 40% formamide, a powerful denaturant, was used to no avail. To determine the role the sequencing reactions might be playing in the artifacts, the cycle sequencing temperatures were increased. The annealing temperature was increased to prevent mis-priming and/or formation of template secondary structure that could cause premature termination. Despite lower DNA banding intensity in the stained gel, the artifact was also present at a correspondingly lower intensity. This result suggests that the sequencing amplification was not the cause of the artifact. This study, however, did not address the possibility that the secondary structure may reside in the population of PCR products. The amplification of the PCR product was done without the 7-deaza-dGTP, a consideration that should be addressed in the future.

It should be kept in mind that the primary objective of the current research was to provide a preliminary study to assess the evolutionary state of the Taxodiaceae family and to provide a sound foundation for future work. Indeed, an accurate, cost-effective methodology was established within the confines of a static portion of DNA. Because of the extremely conserved nature of the 18S gene, however, no statistically valid taxonomical conclusions can be drawn, but the results certainly express some degree of relatedness among the genera. Despite the uniform 18S sequences, the ribosomal cistron can still play an important part in the study of Taxodiaceae. Recent research with spruce (Smith and Klein 1994) has suggested that the ITS-1 region can provide useful phylogenetic information. Future research should, therefore, focus not only on sequencing the ITS-1 region of the genera thus studied, but on the entire Taxodiaceae family.

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APPENDIX A

ISOLATION OF DNA FROM REDWOOD NEEDLES

Procedure

1) For each sample to be extracted, add 0.7 ml of 2X CTAB extraction buffer to a sterile (non-siliconized) 1.5 ml microcentrifuge tube, followed by 35 ml of Proteinase K (10 mg/ml).

   [If preparing 12 samples, transfer 10 ml of CTAB to a sterile 15 ml cell culture tube, followed by 500 ml of Proteinase K (10 mg/ml). Invert to mix, then aliquot 0.736 ml to each of 12 sterile (non-siliconized) 1.5 ml microcentrifuge tubes.]

2) Grind the frozen needles to a powder in a pre-chilled mortar using liquid nitrogen. Powdered tissue may be placed back into a cryotube for future use. **Do not let tissue thaw!!!**

3) Using a sterile round tipped nickel spatula that has been cooled in liquid nitrogen or on dry ice, transfer approximately 100 mg of frozen, powdered tissue into the buffer. (Approximately 2/3 of a spatula tip = 100 mg).

4) Immediately invert the tubes to suspend the tissue in the buffer. Incubate at 65°C for 30 minutes.

5) In a fume hood, add 0.8 ml of chloroform:isoamyl alcohol (24:1) to each sample, mix thoroughly by gentle inversion.

6) Microcentrifuge for 5 minutes at room temperature (RT)

7) Transfer the upper aqueous phase to a sterile 1.5 ml microcentrifuge tube. Discard the tube with the interface and organic phase.

8) Repeat steps 5-7 using a new sterile 1.5 ml microcentrifuge tube.

9) Microcentrifuge the aqueous layer for 5 minutes to pellet any insoluble material.

10) Transfer to a *siliconized* 1.5 ml microcentrifuge tube and be very careful to avoid the insoluble pellet even if it means leaving some of the solution behind.

11) Approximate the volume of the aqueous phase with a pipetter and then add one volume of -20°C isopropanol. Mix thoroughly by gentle inversion and let sit at -20°C for 20 minutes.

12) Microcentrifuge at RT for 10 minutes, remove and discard supernatant. Rinse the pellet with 1 ml of -20°C 70% ethanol (dislodge the pellet from the bottom of the tube with a stream of ethanol from the pipetter).

13) Microcentrifuge at RT for 10 minutes and then rinse with 95% ethanol. (The samples may be stored indefinitely at this point.)

14) Microcentrifuge at RT for 10 minutes, discard the ethanol supernatant and dry the DNA/RNA pellet in a vacuum desiccator for 15 minutes.

15) Add 100 ml of TE (pH 8) and 2 ml of DNase-free RNase A (1mg/ml for a 20 mg/ml final conc.) to the samples and allow to dissolve overnight at 4°C. Alternatively, samples may be incubated at 37°C for 1 hour. Pellet should dissolve easily with minimal flicking.

16) Extract the samples with 100 ml of chloroform:isoamyl alcohol (24:1)to remove RNase.

17) Microcentrifuge for 2 minutes. Transfer the top (aqueous) phase to a sterile, 1.5 ml microcentrifuge tube.

18) Microcentrifuge for 2 minutes to pellet any insoluble material and transfer to a *siliconized* 1.5 ml microcentrifuge tube.

19) Estimate the volume. Add 1/10 volume of 3M sodium acetate (pH 5.5) and 2 volumes of -20°C 95% ethanol. Let sit at -20°C for 20 minutes.

20) Microcentrifuge for 10 minutes at RT, remove and discard supernatant. Wash the pellet with 1ml of -20°C 70% ethanol. (The pellet should be white by this point. If it still retains some off-color, then vacuum desiccate and redissolve in 100 ml of TE (pH 8) overnight at 4°C or at 37°C for 30 minutes. Continue by following steps 18, 19, and 20.

21) Microcentrifuge as before, discard the ethanol supernatant and dry the DNA pellet in a vacuum desiccator for 30 minutes.

22) Dissolve in 100 ml of TE (pH 8) overnight at 4°C or by incubation at 37°C for 30 minutes.

23) Microcentrifuge for 2 minutes to remove any insoluble material and then transfer supernatant to a sterile 0.65 ml microcentrifuge tube.

24) Label the tube with the date, your initials, and any other pertinent information and store at 4°C for a few days or at -20°C for longer periods.

APPENDIX B

PROTOCOL FOR DNA AMPLIFICATION

Procedure

1) Prepare a 20 ng/µl dilution of the stock DNA

DNA stock conc. is _____ ng/µl and _____ µl of 20 ng/µl DNA is needed.
(X µl)(_____ ng/µl stock conc.) = (20 ng/µl)(_____ µl final volume needed)
X=_____ µl stock DNA required to make the final volume of 20 ng/µl DNA.
_____ µl DNA stock (X) + _____ µl sterile H_2O = _____ µl of 20 ng/µl DNA

2) Assemble a master mix to be used for the PCR reaction in a 0.5 ml microcentrifuge tube that is kept on ice. If more than one reaction is to be done, then multiply reagent quantities by the number of reactions.

Master Mix Reagent	Stock concentration	Amount used for 25 µl final volume	Final concentration
Stoffel Buffer	[10X]	2.50 µl	[1X]
dNTP's	[2.5 mM]	2.00 µl	[200 µM]
Primer NS1	[20 µM]	0.50 µl	[0.4 µM]
Primer NS2	[20 µM]	0.50 µl	[0.4 µM]
Taq-Stoffel	[10 U/µl]	0.25 µl	[2.5 U]
$MgCl_2$	[25 mM]	3.00 µl	[3.0 µM]
Final volume		8.75 µl	

3) Prepare the final mix in a 0.5 ml thin-walled PCR tube that is also kept on ice.

Final mix Reagent	Stock concentration	Amount used
DNA	[20 ng/µl]	1.50 µl
Master Mix		8.75 µl
H_2O-Sterile		14.75 µl
Final volume		25.00 µl

4) Place the reaction tubes securely into the thermal cycler so that a minute rim of oil appears at the base of the tube as it is pushed into place. Start the following cycle program.

PCR cycle program

Step 1 - 95°C for 1 min
2 - 95°C for 1 min
3 - 55°C for 1 min
4 - 72°C for 2 min
5 - Go to step 2, 29 X
6 - Hold at 4°C for ∞

APPENDIX C

DIDEOXY CYCLE SEQUENCING PROTOCOL

1) **Add 2 ml of the appropriate d/ddNTP** mix to four 0.5 ml microcentrifuge tubes labeled G,A,T,C and overlay with 10 ml of mineral oil.

2) Prepare the following in a separate 0.5 ml microcentrifuge tube:

SAMPLE REACTION-

Component	Volume (ml)
PCR template (120 fmol)	ml
DNA sequencing buffer (5X)	5.0 ml
NS-1 or 2 Primer diluted 1:10 (4.5 pmol)	2.25 ml
Sterile water	ml
FINAL VOLUME	16.0 ml

3) **Add 1.0 ml of *Taq* DNA polymerase** (5 units/ml) to the primer template mix from step 2 and mix.

4) **Add 4.0 ml of the reaction mix** (from step 3) to the inside wall of each tube containing the d/ddNTP mix (from step 1). Microcentrifuge the tubes for 10 seconds to force all the liquid to the bottom.

5) Place the reaction tubes securely into the thermal cycler so that a minute rim of oil appears at the base of the tube as it is pushed into place. Start one of the following programs based on the primer chosen.

Primer NS-1

Step 1 - 95°C for 2 min
2 - 95°C for 30 sec
3 - 42°C for 30 sec
4 - 70°C for 1 min
5 - Go to step 2, 60 X
6 - Hold at 4°C for ∞

Primer NS-2

Step 1 - 95°C for 2 min
2 - 95°C for 30 sec
3 - 70°C for 30 sec
4 - Go to step 2, 60 X
5 - Hold at 4°C for ∞

6) **Add 3.0 ml of DNA Sequence Stop Solution** (95% formamide and tracking dyes) to the inside wall of each tube after cycling and microcentrifuge for 10 sec to terminate the reactions. Store at -20°C until sequencing gel is ready.

Final reaction volume is 9 ml.

APPENDIX D

SEQUENCING GEL PREPARATION

Plate preparation

1) Clean the plates with alconox, rinse with distilled water and follow with 95% ethanol.

2) Mix 3 µl of Bind Silane with 1 ml of 0.5% glacial acetic acid (5 µl) in 95% ethanol (995 µl). Apply to one side of the short plate with a saturated KimWipe™.

3) After 5 minutes, wipe the plate with 95% ethanol in one direction and then again, perpendicular to the first direction.

4) Repeat step 3 until haze disappears from glass surface, approximately 3-4 times.

5) Change gloves and wipe one side of the long plate with a KimWipe™ wetted with Acrylease™ (2 pumps of the spray bottle or 1 ml).

6) After 5 minutes, wipe the plate with a KimWipe™ lightly dampened with distilled water and follow by buffing the plate with a dry KimWipe™.

7) Change gloves and use the spacers to put together the prepared plates with the treated surfaces facing inward. **Do not allow treated surfaces to contact each other.**

Gel preparation

1) Add 75 ml of millipore water to a 250 ml erhlenmyer flask that has already had 15.4 g urea, 3.67 ml 10X TBE buffer and a bottle of pre-mixed 8% gel components added to it. Bring the volume up to 160 ml with millipore water to make a 6% solution and mix under a vacuum with a magnetic stir bar.

   This solution results in 6% acrylamide with 5% crosslinking and 7 M urea in 1X TBE buffer (52.4 g urea, 7.12 g acrylamide, 0.38 g bis-acrylamide, 1.35 g Tris, 0.69 g boric acid, and 0.104 g disodium EDTA). The bottle can be heated to 37°C to hasten dissolution; however, chill it to below room temperature before the next step to slow down the polymerization, but not so cold that the urea crystallizes.

2) Once the acrylamide is dissolved, a 60 ml syringe that has been fitted with a 2.4 cm GF/A Whatman glass microfiber filter containing apparatus (Millipore, Swinnex-25) on its tip is flushed through its barrel with 20 ml 1X TBE buffer. The acrylamide is then gravity fed in 50 ml increments and subsequently placed under a vacuum for 30 minutes.

3) Withdraw 40 ml of the prepared acrylamide solution with a 60 ml syringe. Through the tip of the syringe add 405 µl freshly prepared 10% ammonium persulfate (0.1 g/1 ml millipore water) and 10 µl of TEMED. Quickly swirl, invert to mix and attach a 1 ml pipet tip to the syringe.

4) With the clamped glass plates propped at a 45° angle back and slightly towards the syringe, slowly add the gel to the lower of the top two corners. Maintain an even flow or else air bubbles might form. If bubbles do form, hit them with a rubber mallet until they escape.

5) When the plates are 3/4 full, start to lay the plates flat and the remaining space will be taken up. With the plates in the flat position, insert the comb flat side down and at an angle at first so that air bubbles may escape. Once all the air has escaped, straighten the comb out and continue to insert it until the bottom side of the two holes on the comb are even with the top edge of the glass plates (6 mm). Fill any gap on either side of the comb with gel until it is once again full. Allow the gel to polymerize for one hour.

6) Following polymerization, attach the gel assembly to the aluminum plate and then to the electrophoresis apparatus. Fill each buffer reservoir with 250 ml 1X TBE and remove any bubbles trapped along the bottom of the gel.

7) Once the top of the gel is submerged, slide out the comb and immediately rinse the top of the gel with the 1X TBE tank buffer. Clean the comb and the outside of the plates to remove excess acrylamide.

8) Connect the leads to the power supply and **pre-electrophorese the gel for 30 minutes at 55 W to warm it up.**

9) While the gel is pre-electrophoresing, thaw (if necessary) the sequencing reactions and keep on ice.

10) After the pre-electrophoresis, rinse the wells with TBE from the upper reservoir to remove any urea that has diffused out of the gel. Reinsert the comb with the tips down so that the points press lightly against the top of the gel without piercing it.

11) Add 125 ml unbuffered 3 M sodium acetate (30.75 g/ 125 ml dH_20) to the lower buffer tank for the counter-ion and 250 ml millipore water to the upper reservoir to make 1/2X TBE. Make sure both tanks are well mixed afterward.

12) Heat samples for 2 minutes at 70°C immediately before loading them onto the sequencing gel.

13) Using the special flat sequencing pipetter tips, load 3 µl of the reaction sample onto the sequencing gel.

14) Run the gel at 33 W (width x length x 0.044 = 33.2 W) until the bromophenol blue dye is near the bottom of the gel.

Gel staining

1) Make the first part of the developing solution (60 g high quality sodium carbonate + 2 L millipore water) and put it in the -20°C freezer (it will be 4-10°C by the time its needed) with a Rubbermade® container.

2) Turn off the power and remove the plate assembly from the apparatus. Remove the tape from the plates and carefully pry the plates apart with a plastic wedge. The gel should adhere to only the short plate.

3) Place the short plate and gel into the fix/stop solution (0.2 L glacial acetic acid + 1.8 L millipore water). Shake gel and solution gently on a rotary shaker at 75 RPM (all shaking should be at 75 RPM) for 20 minutes or until the tracking dyes fade. The gel may be stored overnight in this solution. **Save solution for later use!**

4) Transfer the gel to another tray containing 2 L millipore water and rinse it with shaking for 2 minutes. Repeat 2 more times with fresh water. Lift the plate out and allow the gel to drain for 10-20 seconds before transferring to the next solution.

5) Transfer the gel to a tray containing staining solution (2 g silver nitrate + 3 ml 37% formaldehyde + 2 L millipore water) and shake for 30 minutes.

6) When the staining is nearly finished, complete the developing solution by adding 3 ml of formaldehyde and 0.4 ml of sodium thiosulfate to the 2 L of chilled solution.

7) Transfer the gel to a 2 L millipore water rinse and lightly shake by hand for 15 seconds total (15 seconds from the time of entry until the last drops drain).

8) Immediately transfer it to 2 Liters of 4-10°C developing solution and shake until all the bands are visible or the background is intolerable. This should occur within 6-9 minutes. Be careful, the background will be relatively stable and then darken really quick!!! If no banding has appeared within 5 minutes, make sure the formaldehyde and sodium thiosulfate were added to the developing solution.

9) Stop the development by adding 2 liters of the fix/stop solution from step 3 directly to the developing solution and shake for 3 minutes.

10) Rinse the gel twice with 1 L of millipore water and allow it to dry on the glass plate at room temperature in a dust-free environment.